AVIAN DISEASES 54:857–861, 2010

Colonization of Avian Reproductive-Tract Tissues by Variant Subpopulations of *Salmonella* Enteritidis

Jean Guard,[A] Richard K. Gast, and Rupa Guraya

U.S. Department of Agriculture, Athens, GA 30677

Received 22 September 2009; Accepted and published ahead of print 18 January 2010

SUMMARY. Leghorn hens were infected with *Salmonella* Enteritidis cultures of known genomic content and subpopulation characteristics to determine comparative abilities to colonize the avian reproductive tract. Group 1 received phage-type (PT)4 22079, which is a dimorphic subpopulation that can both contaminate eggs and form biofilm. Group 2 received a 90:10 mixture of monomorphic PT13a strains 21027 and 21046, which produce biofilm or contaminate eggs, respectively. Group 3 received a 10:90 mixture of the same two PT13a strains, respectively. Trials were repeated three times and a total of 30 hens per treatment group were infected. Dosage was by oral gavage and was calculated as $8.6 \pm 2.01 \times 10^7$ colony-forming units per hen. Liver, spleen, and three different sections of oviduct (ovary, upper oviduct, and lower oviduct) were cultured per bird. Results were that all three groups had livers and spleens that were mostly positive (90.0% and 94.4% of 270 hens cultured, respectively). Reproductive-tract organs yielded 75 positives from 270 hens (27.8%), and treatment groups ranged from a low of 6.7% to a high of 76.7% positive cultures in any one trial. There was no significant difference between the numbers of positive reproductive-tract samples between treatment groups due to variance. These results suggest that the status of the reproductive tract at the time of infection may impact recovery of culture-positive tissue and contribute to variance. It is suggested that *Salmonella* Enteritidis cultures that vary in subpopulation composition have subtle differences in colonization of reproductive tissue that contribute to variance in egg contamination. Culture of non–reproductive-tract organs such as the liver and spleen was overall more reliable for detection of infected hens. The spleen was especially useful for detection because of its small size. Further research is needed to determine how sex hormones influence the infection pathway that results in egg contamination.

RESUMEN. Colonización en los tejidos del tracto reproductor por subpoblaciones variantes de *Salmonella* Enteritidis.

Gallinas tipo Leghorn fueron infectadas con cultivos de *Salmonella* Enteritidis que contenían características genomicas y subpoblaciones conocidas para comparar la capacidad para colonizar el tracto reproductivo de aves. El Grupo 1 recibió el inóculo PT4 22079, que es una subpoblación dimórfica que puede contaminar huevos y formar biopelículas. Grupo 2 recibió una mezcla 90:10 de las cepas monomórficas PT13a; 21027 y 21046, que producen biopelículas o pueden contaminar huevos, respectivamente. El grupo 3 recibió una mezcla 10:90 con las mismas cepas PT13a, respectivamente. Los ensayos se repitieron tres veces y se infectaron un total de 30 gallinas por tratamiento. Las dosis se aplicaron por sonda oral y se calculó fue de $8.6 \pm 2.01 \times 10^7$ unidades formadoras de colonias por gallina. Se cultivaron hígado, bazo, y tres secciones diferentes de oviducto (ovario, y partes superior e inferior del oviducto) por ave. Los resultados fueron que los tres grupos de aves mostraron hígados y bazos que eran en su mayoría positivos (90.0% y 94.4% de 270 gallinas cultivadas, respectivamente). Los órganos reproductores mostraron 75 muestras positivas de 270 gallinas (27.8%) y los grupos tratados oscilaron de un mínimo de 6.7% a un máximo de 76.7% de cultivos positivos en cualquiera de los experimentos. No hubo diferencia significativa en el número de muestras positivas del tracto reproductivo entre los grupos de tratamiento debido a la varianza. Estos resultados sugieren que la situación del aparato reproductor en el momento de la infección puede afectar la recuperación de cultivos positivos de los tejidos y contribuir en la varianza. Se sugiere que los cultivos de *Salmonella* Enteritidis que varían en la composición de sus subpoblaciones tienen diferencias sutiles en la colonización de los tejidos reproductivos lo que contribuye a la variación en la contaminación de los huevos. El cultivo de órganos no reproductivos como el hígado y el bazo resultó en general más confiable para la detección de las gallinas infectadas. El bazo es especialmente útil para la detección debido a su tamaño pequeño. Se necesita más investigación para determinar cómo las hormonas sexuales influyen en el desarrollo de la infección que provoca la contaminación de los huevos.

Key words: *Salmonella enterica*, eggs, food safety, poultry, pathogen, reproduction

Abbreviations: BF = biofilm forming; BG = brilliant green; BHI = brain–heart infusion; EC = egg contaminating; LPS = lipopolysaccharide; PBS = phosphate-buffered saline; PT = phage type; SNP = single-nucleotide polymorphism

Salmonella enterica subsp. I serovar Enteritidis (*Salmonella* Enteritidis) is the world's leading cause of human salmonellosis (3,4,5,9,10,17,24,29). It is unique among over 1400 *Salmonella enterica* subsp. I serotypes, because it alone has the ability to contaminate the internal contents of eggs produced by otherwise healthy hens at a frequency that is a major source of food-borne disease (26,27). The phenomenon of egg contamination appears to be a pinnacle biological behavior impacting epidemiologic prevalence, because *Salmonella* Enteritidis has remained a prevalent and persistent problem within the food supply ever since its emergence. The presence of *Salmonella* Enteritidis within broilers is also a major concern (1). Finding *Salmonella* Enteritidis in chicken flocks can be especially problematic for exporters of live breeding stock if birds test positive for the pathogen when received by the importer.

Progress has been made in understanding some of the more confounding aspects of this serotype. For example, strains of *Salmonella* Enteritidis vary greatly in their ability to contaminate eggs (6,7,8,12,20,25), even when they appear to be highly clonal (15,16,19,22). An explanation for this conundrum has been provided. *Salmonella* Enteritidis strains that vary in the ability to contaminate eggs can have less than 0.01% difference in genome content (http://www.ncbi.nlm.nih.gov/genomes/static/Salmonella_SNPS.html). Most of these small-scale evolutionary events are not

[A]Corresponding author. E-mail: jean.guard@ars.usda.gov

readily detectable by typing methods; for example, pulsed field gel electrophoresis detected only two enzyme restriction sites that varied between strains that had a total of 247 differentiating evolutionary events (15). *Salmonella* Enteritidis is different from serotypes associated with carcass contamination, because it readily produces a capsule-like structure composed of the O-antigen region of lipopolysaccharide (LPS). The O-antigen capsule imparts tropism for the avian reproductive tract and mitigates signs of disease in the infected hen (21). Transcriptome analysis has shown that increased expression of one LPS gene, namely *rfbH*, was linked to long-term survival of the pathogen in eggs (6,8). However, transcriptome analysis did not necessarily track evolution linked to capsule formation, because amino acid changes were detected within two other genes of the rfb operon, namely *rfbB* and *rfbX* (http://www.ncbi.nlm.nih.gov/genomes/static/Salmonella_SNPS.html).

Although progress has been made in understanding the type of evolutionary events linked to the emergence of egg contamination, hen infection studies do not routinely incorporate new knowledge of the subpopulation biology of *Salmonella* Enteritidis into the design of challenge experiments (11). This is an important issue, because questions about how to detect *Salmonella* Enteritidis within flocks must be addressed in order for producers to implement new regulations (2). In this article we investigate the hypothesis that variation in bacterial loads in different tissues of hens exists, and that some tissues are better for sampling than others. To investigate this hypothesis, three different groups of hens were challenged with a similar dose of *Salmonella* Enteritidis that had different compositions of subpopulations. A dimorphic strain of phage-type (PT)4 *Salmonella* Enteritidis that forms biofilm (biofilm-forming, BF) and contaminates eggs (egg contaminating, EC) (BF+EC+) was used to infect the first group of 10 hens. Two monomorphic PT13a strains designated as BF+EC− and BF−EC+, which either form biofilm or contaminate eggs but not both, were used to infect another two groups of hens at percentage volume:volume ratios of 90:10 and 10:90, respectively. Results strongly suggest that considerable differences exist between bacterial loads of *Salmonella* Enteritidis in tissues and that knowledge of these differences could be used to improve detection.

MATERIALS AND METHODS

Bacterial strains and characterization. *Salmonella enterica* subsp. I serovar Enteritidis with in-house accession numbers 22079, 21046, and 21027 were used throughout experiments. Strains were either in the PT 4 lineage (PT4 22079), which is characterized by lysogenized mosaic phage ST64b, or in the Fels2 lineage, namely, PT13a 21027 and PT13a 21046 (18,23). The whole genomes of all three strains were compared to the Sanger Institute reference genome for PT4 *Salmonella* Enteritidis strain P125109 (GenBank: AM933172) by mutational mapping with the use of computer-generated primers (NimbleGen, Madison, WI) (28). For purposes of identification, base pair 3973601 (SEN_r012, a 23S ribosomal gene) differentiates PT4 22079 from the reference strain of the Sanger Institute because of a G to C substitution (unpublished data). A list of single-nucleotide polymorphisms (SNPs) that differentiate the two PT13a strains from each other as well as at least one of them from reference strain P125109 is publically available at http://www.ncbi.nlm.nih.gov/genomes/static/Salmonella_SNPS.html. All three strains were previously characterized by phenotype microarray (Biolog, Hayward, CA), which showed that PT4 22079 had metabolic characteristics intermediate to that of PT13a 21027 and PT13a 21046 (18). PT4 22079 was isolated from a stream downstream of flocks producing contaminated eggs during outbreaks in California (courtesy of H. Kinde). Strains PT13a 21027 and PT13a 21046 were both isolated from the spleen of a mouse that was naturally infected and caught on-farm during outbreaks of egg contamination in Pennsylvania (courtesy of D. Henzler).

Inoculum preparation. Strains were reconstituted from culture frozen in brain–heart infusion (BHI) broth supplemented with 20% glycerol and stored at −80 C. Frozen stocks were streaked onto brilliant green (BG) agar (Acumedia, Lansing, MI) for colony isolation. Plates were incubated for 16 hr at 37 C. Single colonies of each strain were transferred to 10 ml BHI and incubated for 24 hr at 37 C. Cultures were centrifuged at 10,000 rpm for 10 min and then suspended in 1× phosphate-buffered saline (PBS) to an optical density of 1.0 at 600 nm (AD_{600}). Cultures used to infect birds were made by a final 10-fold dilution in 1× PBS; however, PT13a 21027 and 21046 cultures were mixed, respectively, to produce 90:10 and 10:90 volume:volume (vol:vol) inocula for infecting different groups of birds in each trial. To determine dose, an aliquot from each inoculum was serially diluted fivefold for five dilutions. Then 20 µl per well was plated onto BG agar to determine plate counts and standard deviations.

Evaluation of colony phenotype. Previous analysis of colony morphology showed that PT4 22079 is BF+EC+, PT13a 21027 is BF+EC−, and PT13a 21046 is BF−EC+. To evaluate the ratio of colonies that were positive and negative for biofilm formation in all three preparations at the time of dosing, inocula were streaked on BG agar for isolation of individual colonies and incubated at 37 C for 16 hr. Ten colonies per inoculum were spot transferred to a single BG plate in a pattern to separate the next-generation colonies maximally. Ten plates were prepared for each inoculum so that a total of 100 well-spaced colony-forming units could be evaluated for each inoculum. Plates were incubated at 37 C for 16 hr and then transferred to ambient temperature for the next 5 days (120 hr). To avoid desiccation of agar, plates were put into plastic containers with moist paper towels and tight-fitting lids. Colony morphologies were recorded before, during, and every 24 hr after incubation at ambient temperature by digital imaging (Biorad imager and software; Hercules, CA), and were tabulated.

Experimental infection of laying hens. All animal experiments were reviewed for appropriate care and use in research by the Institutional Animal Care and Use Committee. In each of three trials, 40 laying hens were obtained from the specific-pathogen-free flock of single-comb white leghorn chickens (negative for antibodies to *Salmonella* in periodic routine monitoring) at the Southeast Poultry Research Laboratory in Athens, GA. These hens (27, 33, and 35 wk old at the beginning of the first, second, and third trials, respectively) were distributed among four separately housed groups (three inoculated groups of 12 birds each and an uninfected negative control group of 4 birds) in a disease-containment facility. Birds were housed in individual laying cages and provided with water and pelleted feed *ad libitum*. Inoculated hens received a 1-ml oral dose of the appropriate *Salmonella* Enteritidis culture preparation.

Fecal samples. Immediately before inoculation, sterile cotton swabs were used to collect samples of voided feces from polystyrene trays (food-grade but not sterile) placed under each cage. These samples were transferred to 9 ml of tetrathionate broth (Oxoid, Cambridge, UK) and incubated for 24 hr at 37 C. A 10-µl portion from each broth culture was then streaked onto BG agar (Becton, Dickinson, and Co., Franklin Lakes, NJ) supplemented with 0.02 mg/ml of novobiocin (Sigma Chemical Co., St. Louis, MO) and incubated for 24 hr at 37 C. The identity of presumptive colonies of *Salmonella* Enteritidis was confirmed biochemically and serologically (30).

Internal organ samples. At 5 days postinoculation in each trial, all hens were humanely euthanatized to allow removal of internal tissues for bacteriologic culture. Portions (approximately 5–10 g) of the liver, spleen, ovary, upper oviduct (centered on the infundibulum/magnum junction), and lower oviduct (centered on the isthmus/uterus junction) from each hen were aseptically removed, transferred to 50 ml of tetrathionate broth, and mixed by stomaching for 30 sec. Each broth culture was incubated for 40 hr at 37 C, and a 10-µl aliquot was then streaked onto BG agar plus novobiocin. After incubation of these plates for 24 hr at 37 C, typical *Salmonella* Enteritidis colonies were subjected to biochemic and serologic confirmation.

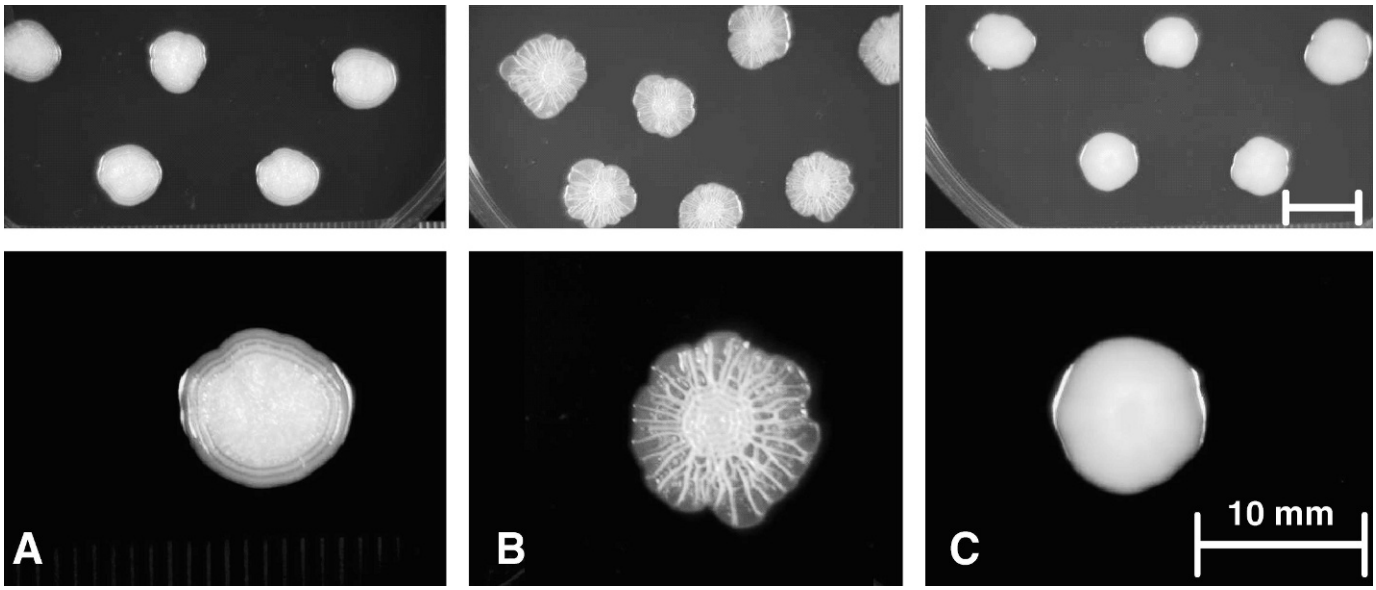

Fig. 1. Prominent colony morphologies of *Salmonella enterica* serovar Enteritidis. Top panels: multiple colonies of each strain, 10 per plate, were grown for 120 hr at ambient temperature following a 16-hr incubation at 37 C. Bottom panels: magnified view of representative colonies. Inset marker is 10 mm. (A) *Salmonella* Enteritidis PT4 22079, with weak slow biofilm formation; (B) *Salmonella* Enteritidis PT13a 21027, with strong rapid biofilm formation; (C) *Salmonella* Enteritidis PT13a 21046, with no discernible biofilm.

Statistical analysis. For each trial, significant differences ($P < 0.05$) in the frequency of *Salmonella* Enteritidis recovery from internal organs between treatment groups were determined by Fisher's exact test. Data were analyzed with Instat biostatistics software (GraphPad Software, San Diego, CA). Nonparametric analysis of variance (ANOVA) of variation between treatments in producing positive organs was conducted with the use of the Kruskal–Wallis test (GraphPad Software).

RESULTS

Characteristics of inocula A, B, and C. Analysis of colony morphology of all 3 strains used to prepare inocula showed that PT4 22079 formed a biofilm that was not as distinctive as the one formed by strain PT13a 21027; in contrast, PT13a 21046 did not form biofilm (Fig. 1). When PT13a 21027 and PT13a 21046 were mixed to achieve contrasting vol:vol ratios of 90:10 (treatment B) and 10:90 (treatment C), inocula were obtained that met experimental objectives of infecting birds with cultures heavily skewed toward one phenotype (Table 1).

Colonization of reproductive-tract samples. Hens infected by treatments A, B, and C, with three trials per treatment, gave results as shown in Table 2. Reproductive-tract tissue had a wide range of results, regardless of anatomic location. For example, ovarian tissue ranged from no positive samples for a group of 10 hens to positive results from 9 of 10 hens cultured (Table 2, treatment A, trial iii). For this treatment group, trial iii had only one positive sample per 10 birds from culture of upper and lower oviduct. The uniformity of results across three different locations within the reproductive tract suggests that colonization was markedly lower in trial iii following infection with the PT4 strain; in other words, results were probably not due to a sampling error. Although hens infected with PT4 22079 had the widest range of results from reproductive-tract tissue for the three treatment groups, this group also produced the highest percent positive reproductive-tract samples overall. Namely, 40% (36/90) of all reproductive-tract samples cultured were positive for *Salmonella* Enteritidis following treatment A. Treatment B, which was the 90:10 ratio of PT13a strains, yielded 27.7% (25/90) culture positive reproductive-tract samples. Treatment C, which was the 10:90 ratio of PT13a strains, yielded 20% (18/90) culture positive reproductive-tract samples. Statistical analysis suggested that treatment C did not fit a Gaussian (normal) distribution in comparison to treatments A and B; however, the P value used to determine the normality of distribution was borderline (P value = 0.044). Analysis of results using both parametric one-way and nonparametric ANOVA indicated that none of the treatment groups were statistically different in regards to the number of positive reproductive-tract samples across all trials (all P values > 0.05). However, analysis of variance with the chi-square test for independence indicated that the variance observed in reproductive-tract tissue between treatment A (monoculture of PT4 22079) and treatment C (10:90 ratio of PT13a strains 21027 and 21046) was significant (P value < 0.01).

In contrast to the extremes of variation observed from culturing reproductive-tract tissue, liver and spleens were mostly positive. Treatment group C, infected with the PT13a 21027:21046 at a ratio of 10:90, produced 100% positive livers and spleens in spite of producing the lowest percentage of positive reproductive-tract samples. In contrast, group A, which was infected with PT4 22079, had 27 culture positive liver samples and 26 positive liver samples from 30 birds (90% and 87% positive, respectively). Group B, infected with the 90:10 mix of PT3a strains, had 26 and 27 (87% and 90%) positive liver and spleen samples. There was no statistical difference in the number of positive samples. The absence of variation in results for Treatment C prevents assessment of variance, which appears from raw data to be negligible.

Table 1. Colony phenotype percentages per treatment group (biofilm positive:biofilm negative).

	A (22079)	B (21027:21046)	C (21027:21046)
Trial 1	100:0	92:8	15:85
Trial 2	100:0	71:29	11:89
Trial 3	100:0	91:9	4:96

Table 2. Results from culturing organs of hens infected with *Salmonella enterica* serovar Enteritidis.

Organ and trial number	% Culture positive organs (treatment)[A,B]		
	A	B	C
Liver			
i	100	90	100
ii	100	80	100
iii	70	90	100
Spleen			
i	100	90	100
ii	90	90	100
iii	70	90	100
Ovary			
i	90	30	20
ii	60	50	40
iii	0	50	40
Upper oviduct			
i	80	0	10
ii	20	40	20
iii	10	10	20
Lower oviduct			
i	70	30	0
ii	20	40	20
iii	10	0	10

[A]Treatment A was inoculum prepared from *Salmonella* Enteritidis PT4 22079; B was a 90:10 ratio of PT13a 21027 and PT13a 21046, respectively; C was a 10:90 ratio of the two PT13a strains, respectively. See text and Fig. 1 for phenotypic characteristics of each strain.

[B]Average dose per bird was $8.6 \pm 2.01 \times 10^7$ colony-forming units.

DISCUSSION

The spleen appears to be an excellent organ to culture for the presence of *Salmonella* Enteritidis. Although the liver appears just as likely to yield a positive culture, the spleen is small and can be cultured in its entirety and in a way that more completely releases intracellular bacteria. For example, previous experiments have disrupted spleen cells within a small volume by vortexing with glass beads (11). The liver of a mature bird is more difficult to process, because it is much larger and it has a collagen architecture that can prevent cell disruption. Culturing of spleens from a subset of birds bound for export could aid in assuring both exporter and importer that the flock is free of *Salmonella* Enteritidis. Because foundation lineage birds used to establish new production flocks are of high individual value, it is possible that sentinel birds of low value could be implanted into flocks for testing for the presence of *Salmonella* Enteritidis in organs.

The reproductive tract may not be an appropriate target for testing for *Salmonella* Enteritidis. It is a large organ and culturing any one section of the reproductive tract may yield a negative result. In addition, there may be hormonal-cycle–dependent resistance to colonization. It is suggested that subpopulation characteristics of *Salmonella* Enteritidis facilitate egg contamination by extending the phenotypic repertoire of the pathogen to overcome hormonal-dependent resistance that is associated with different stages of the reproductive cycle. The different phage-type lineages of *Salmonella* Enteritidis, namely, the PT4 and PT13a/PT8 lineages, may have subtle differences in regards to which stage of the hormonal cycle is best for achieving colonization. Results suggest that dimorphic cultures of PT4 yield exceptional variance but higher peak loads in reproductive niches. Previous experimentation had suggested that BF strains of *Salmonella* Enteritidis were more skewed to efficient colonization of the intestinal tract of the orally infected hen (14). In fact, *Salmonella* Enteritidis that forms the type of highly organized biofilm observed for PT13a 21027 has never been recovered from the internal contents of the egg across multiple experiments. PT13a 21046 lost the ability to make biofilm, but retained the ability to make capsular-like lipopolysaccharide, to grow to high cell density, and to make capsular-like lipopolysaccharide. It has been efficient at egg contamination, but more so by the systemic than the oral route of challenge (11,12,13).

It is common to find a mixture of phenotypes in cultures of *Salmonella* Enteritidis, which suggests that there may be some synergy associated with mixed cultures for achieving egg contamination. For example, the mixed phenotype PT13a/PT8 lineage may be more successful at colonization under a broader range of hormonal parameters, but at the price of not reaching the peak loads of the PT4 lineage. Alternatively, the biofilm-forming subpopulation may introduce a nidus of inflammation in the intestinal tract that is a portal for strains more adapted to systemic niches following oral colonization. These findings may have implications for broiler carcass contamination. For example, a strain of *Salmonella* Enteritidis that achieves more efficient colonization of the intestinal tract might be more likely to cause carcass contamination. There is evidence that contamination of broiler carcasses increased in incidence within the United States in recent years (1). More research is needed to compare strains of known genomic content that vary in the ability to contaminate eggs and that otherwise threaten the safety of the food supply.

REFERENCES

1. Altekruse, S. F., N. Bauer, A. Chanlongbutra, R. DeSagun, A. Naugle, W. Schlosser, R. Umholtz, and P. White. *Salmonella enteritidis* in broiler chickens, United States, 2000–2005. Emerg. Infect. Dis. 12:1848–1852. 2006.

2. Anonymous. Prevention of *Salmonella enteritidis* in shell eggs during production, storage, and transportation. Final rule. Fed. Regist. 74:33029–33101. 2009.

3. Barrow, P. A. The paratyphoid salmonellae. Rev. Sci. Tech. Off. Int. Epizoot. 19:351–375. 2000.

4. Braden, C. R. *Salmonella enterica* serotype Enteritidis and eggs: a national epidemic in the United States. Clin. Infect. Dis. 43:512–517. 2006.

5. Durecko, R., D. Saladiova, P. Popelka, and I. Simanska. Epidemiological and epizootological aspects of salmonellosis. Bratisl. Lek. Listy. 105:414–418. 2004.

6. Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, R. Gast, T. J. Humphrey, and F. Van Immerseel. Mechanisms of egg contamination by *Salmonella* Enteritidis. FEMS Microbiol. Rev. 33:718–738. 2009.

7. Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, and F. Van Immerseel. *Salmonella enterica* serovar Enteritidis genes induced during oviduct colonization and egg contamination in laying hens. Appl. Environ. Microbiol. 74:6616–6622. 2008.

8. Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, and F. Van Immerseel. The *Salmonella* Enteritidis lipopolysaccharide biosynthesis gene rfbH is required for survival in egg albumen. Zoonoses Public Health 56:145–149. 2009.

9. Gillespie, I. A., S. J. O'Brien, G. K. Adak, L. R. Ward, and H. R. Smith. Foodborne general outbreaks of *Salmonella* Enteritidis phage type 4 infection, England and Wales, 1992–2002: where are the risks? Epidemiol. Infect. 133:795–801. 2005.

10. Greig, J. D., and A. Ravel. Analysis of foodborne outbreak data reported internationally for source attribution. Int. J. Food Microbiol. 130:77–87. 2009.

11. Guard-Bouldin, J., R. K. Gast, T. J. Humphrey, D. J. Henzler, C. Morales, and K. Coles. Subpopulation characteristics of egg-contaminating

Salmonella enterica serovar Enteritidis as defined by the lipopolysaccharide O chain. Appl. Environ. Microbiol. 70:2756–2763. 2004.

12. Guard-Petter, J. Variants of smooth *Salmonella enterica* serovar Enteritidis that grow to higher cell density than the wild type are more virulent. Appl. Environ. Microbiol. 64:2166–2172. 1998.

13. Guard-Petter, J. The chicken, the egg and *Salmonella enteritidis*. Environ. Microbiol. 3:421–430. 2001.

14. Guard-Petter, J., L. H. Keller, M. M. Rahman, R. W. Carlson, and S. Silvers. A novel relationship between O-antigen variation, matrix formation, and invasiveness of *Salmonella enteritidis*. Epidemiol. Infect. 117:219–231. 1996.

15. Hudson, C. R., M. Garcia, R. K. Gast, and J. J. Maurer. Determination of close genetic relatedness of the major *Salmonella enteritidis* phage types by pulsed-field gel electrophoresis and DNA sequence analysis of several Salmonella virulence genes. Avian Dis. 45:875–886. 2001.

16. Liebana, E., L. Garcia-Migura, J. Guard-Petter, S. W. McDowell, S. Rankin, H. M. Opitz, F. A. Clifton-Hadley, and R. H. Davies. *Salmonella enterica* serotype Enteritidis phage types 4, 7, 6, 8, 13a, 29 and 34: a comparative analysis of genomic fingerprints from geographically distant isolates. J. Appl. Microbiol. 92:196–209. 2002.

17. Little, C. L., S. Surman-Lee, M. Greenwood, F. J. Bolton, R. Elson, R. T. Mitchell, G. N. Nichols, S. K. Sagoo, E. J. Threlfall, L. R. Ward, I. A. Gillespie, and S. O'Brien. Public health investigations of *Salmonella* Enteritidis in catering raw shell eggs, 2002–2004. Lett. Appl. Microbiol. 44:595–601. 2007.

18. Morales, C. A., S. Porwollik, J. G. Frye, H. Kinde, M. McClelland, and J. Guard-Bouldin. Correlation of phenotype with the genotype of egg-contaminating *Salmonella enterica* serovar Enteritidis. Appl. Environ. Microbiol. 71:4388–4399. 2005.

19. Pang, J. C., T. H. Chiu, C. S. Chiou, A. Schroeter, B. Guerra, R. Helmuth, and H. Y. Tsen. Pulsed-field gel electrophoresis, plasmid profiles and phage types for the human isolates of *Salmonella enterica* serovar Enteritidis obtained over 13 years in Taiwan. J. Appl. Microbiol. 99:1472–1483. 2005.

20. Parker, C., K. Asokan, and J. Guard-Petter. Egg contamination by *Salmonella* serovar Enteritidis following vaccination with Delta-aroA Salmonella serovar typhimurium. FEMS Microbiol. Lett. 195:73–78. 2001.

21. Parker, C. T., B. Harmon, and J. Guard-Petter. Mitigation of avian reproductive tract function by *Salmonella enteritidis* producing high-molecular-mass lipopolysaccharide. Environ. Microbiol. 4:538–545. 2002.

22. Peters, T. M., C. Berghold, D. Brown, J. Coia, A. M. Dionisi, A. Echeita, I. S. Fisher, A. J. Gatto, N. Gill, J. Green, P. Gerner-Smidt, M. Heck, I. Lederer, S. Lukinmaa, I. Luzzi, C. Maguire, R. Prager, M. Usera, A. Siitonen, E. J. Threlfall, M. Torpdahl, H. Tschape, W. Wannet, and W. K. Zwaluw. Relationship of pulsed-field profiles with key phage types of *Salmonella enterica* serotype Enteritidis in Europe: results of an international multi-centre study. Epidemiol. Infect. 135:1274–1281. 2007.

23. Porwollik, S., C. A. Santiviago, P. Cheng, L. Florea, S. Jackson, and M. McClelland. Differences in gene content between *Salmonella enterica* serovar Enteritidis isolates and comparison to closely related serovars Gallinarum and Dublin. J. Bacteriol. 187:6545–6555. 2005.

24. Przybylska, A. Foodborne infections and intoxications in Poland in 2002. Przegl. Epidemiol. 58:85–101. 2004.

25. Rahman, M. M., J. Guard-Petter, and R. W. Carlson. A virulent isolate of *Salmonella enteritidis* produces a *Salmonella typhi*-like lipopolysaccharide. J. Bacteriol. 179:2126–2131. 1997.

26. Sharp, J. C. Salmonellosis and eggs. Br. Med. J. 297:1557–1558. 1988.

27. St. Louis, M. E., D. L. Morse, M. E. Potter, T. M. DeMelfi, J. J. Guzewich, R. V. Tauxe, and P. A. Blake. The emergence of grade A eggs as a major source of *Salmonella enteritidis* infections. New implications for the control of salmonellosis. JAMA 259:2103–2107. 1988.

28. Thomson, N. R., D. J. Clayton, D. Windhorst, G. Vernikos, S. Davidson, C. Churcher, M. A. Quail, M. Stevens, M. A. Jones, M. Watson, A. Barron, A. Layton, D. Pickard, R. A. Kingsley, A. Bignell, L. Clark, B. Harris, D. Ormond, Z. Abdellah, K. Brooks, I. Cherevach, T. Chillingworth, J. Woodward, H. Norberczak, A. Lord, C. Arrowsmith, K. Jagels, S. Moule, K. Mungall, M. Sanders, S. Whitehead, J. A. Chabalgoity, D. Maskell, T. Humphrey, M. Roberts, P. A. Barrow, G. Dougan, and J. Parkhill. Comparative genome analysis of *Salmonella* Enteritidis PT4 and *Salmonella* Gallinarum 287/91 provides insights into evolutionary and host adaptation pathways. Genome Res. 18:1624–1637. 2008.

29. Todd, E. C. Epidemiology of foodborne diseases: a worldwide review. World Health Stat. Q. 50:30–50. 1997.

30. Waltman, W. D., and R. K. Gast. Salmonellosis. In: A laboratory manual for the isolation and identification of avian pathogens, 5th ed. L. Dufour-Zavala, D. E. Swayne, J. R. Glisson, J. E. Pearson, W. M. Reed, M. W. Jackwood, and P. R. Woolcock, eds. American Association of Avian Pathologists, Athens, GA. pp. 3–9. 2008.